Synthesis of peptidoglycan fragments and evaluation of their biological activity

Seiichi Inamura,[a] Yukari Fujimoto,[a] Akiko Kawasaki,[a] Zenyu Shiokawa,[a] Eva Woelk,[b] Holger Heine,[b] Buko Lindner,[c] Naohiro Inohara,[d] Shoichi Kusumoto[a] and Koichi Fukase*[a]

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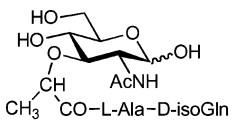
The peptidoglycan (PG) bacterial cell wall glycoconjugate has been well known as a strong immunopotentiator. Partial structures of PG were chemically synthesized for elucidation of precise biological activities. Effective construction of distinct repeating glycans of PG was accomplished by the coupling of a key disaccharide glucosaminyl-β(1–4)-muramic acid unit. Stereoselective glycosylation of disaccharide units was achieved by neighboring group participation of the *N*-Troc (Troc = 2,2,2-trichloroethoxycarbonyl) group and appropriate reactivity of *N*-Troc-glucosaminyl trichloroacetimidate. By using an efficient synthetic strategy, mono-, di-, tetra- and octasaccharide fragments of PG were synthesized in high yields. The biological activity of synthetic fragments of PG was evaluated by induction of tumor necrosis factor-α (TNF-α) from human monocytes, and toll-like receptor 2 (TLR2) and Nod2 dependencies by using transfected HEK293 cells, respectively. Here we reveal that TLR2 was not stimulated by the series of synthetic PG partial structures, whereas Nod2 recognizes the partial structures containing the MDP moiety.

Introduction

The first line of defence against microorganisms is innate immunity with molecular recognition of common bacterial components such as peptidoglycan (PG), lipopolysaccharide (LPS), lipoproteins and bacterial DNA. These components induce many kinds of mediator such as cytokines, prostaglandins, the platelet activating factor, and NO.[1,2,3,4] The mediators stimulate immune competent cells and, consequently, the above bacterial components show immunopotentiating activity. Peptidoglycan (PG) has been well-known as a potent immunopotentiator[5] and numerous studies have been performed to clarify the mechanism of stimulating the immune system. However, the immunostimulating mechanism of PG has not been well understood. The present work aims to clarify this mechanism by using definite PG partial structures, which are chemically synthesized.

Muramyl dipeptide (MDP) 1

PG consists of polysaccharide chains linked to a peptide network to form a three-dimensional rigid structure constructing a bacterial cell wall. The polysaccharide chain is β(1–4) glycan composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) of which the carboxyl group is the point of linkage to the peptide. Two research groups, including ourselves, have independently demonstrated that the minimum structure required for the immunostimulation is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide: MDP) **1**.[6,7,8]



Muramyl dipeptide (MDP) **1**

Recent studies revealed that toll-like receptors (TLRs) play a crucial role in innate immunity by recognizing microbial components and activating immune cells against microbes. Currently, eleven TLRs have been identified.[9] The different TLRs are activated by different types of microbial components. Since microbial components isolated from natural sources are usually contaminated with other microbial components, their biological activities, especially their receptors, must be verified by using synthetic specimens. TLR4 proved to be the receptor for lipopolysaccharide (LPS).[10] TLR9 is responsible for the recognition of the CpG sequence of bacterial DNA.[11] TLR2 is implicated in immunostimulation by PG, lipoprotein (BLP), and lipoarabinomannan, though only BLP proved to be the ligand of TLR2 by using synthetic lipopeptides, partial structures of BLP.[12]

Recently, Boneca *et al.* reported that highly purified PGs are not sensed by TLR2.[13] On the other hand, Dziarski's group recently re-evaluated the fact that peptidoglycan is a toll-like receptor 2 activator,[14] and this is still controversial.

[a]*Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka, 560-0043, Japan. E-mail: koichi@chem.sci.osaka-u.ac.jp; Fax: +81-6-6850-5391; Tel: +81-6-6850-5419*
[b]*Department of Immunology and Cell Biology, Research Center Borstel, 23845, Borstel, Germany*
[c]*Department of Immunochemistry and Biochemical Microbiology, Research Center Borstel, 23845, Borstel, Germany*
[d]*Department of Pathology, The University of Michigan Medical School, Ann Arbor, Michigan, 48109, USA*
† Present address: Suntory Institute for Bioorganic Research, Shimamoto-cho, Mishima-gun, Osaka 618–8503, Japan, and Department of Environmental and Biotechnological Frontier Engineering, Fukui University of Technology, Fukui, Fukui 910-8505, Japan.

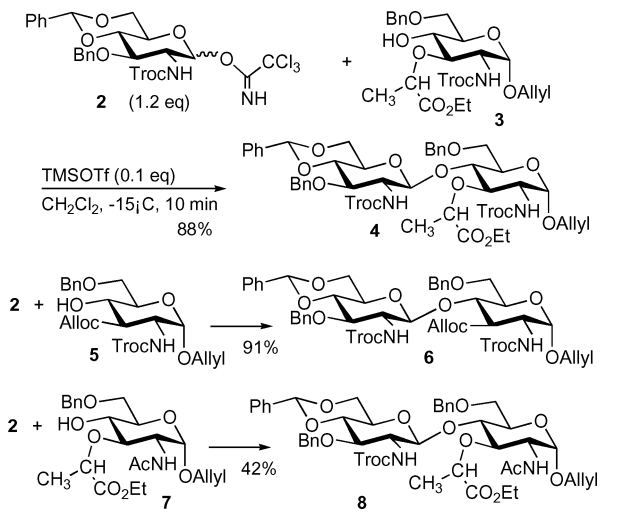
Membrane CD14 (mCD14) is a glycosylphosphatidylinositol-anchored protein expressed on macrophage/monocyte and also plays an important role in the recognition of microbial components. It binds PG and other microbial components such as LPS, and then transports them to their respective receptors.[15] Recent studies, however, indicate that MDP exhibited activity in a CD14- and TLR2-independent manner.[16,17,18]

In addition, the activity of MDP was found to be not identical with that of PG. Soluble peptidoglycan (sPG) obtained from enzyme treatment of PG exhibited stronger immunostimulating activity than MDP.[15] The degree of cross-linking of the peptide in sPG is low and, hence, sPG has a shorter peptide chain but still has long glycan chains. On the other hand, a glycosidase (=muramidase) digested mixture of sPG showed lower activity.[19] These studies indicated that the glycan chain length might be important for the activity. In the present study, therefore, we synthesized various peptidoglycan partial structures with a longer glycan chain, *i.e.*, tetrasaccharides having di-, tri-, tetra-, and pentapeptide units, and octasaccharide having dipeptide units, and evaluated their biological activities and checked CD14 and TLR2 dependency. We also synthesized disaccharide analogues with newer methods. Mobashery *et al.* also recently reported the synthesis of PG partial structures composed of tetrasaccharide.[20]

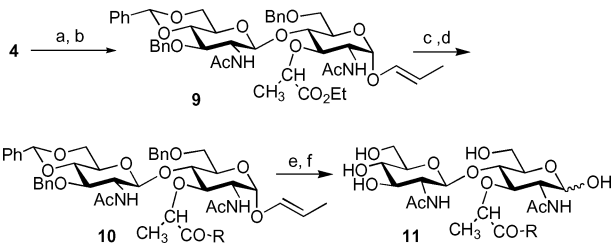
Results and discussion

For the effective construction of distinct repeating glycans of PG, we applied a sequential coupling reaction of a key disaccharide glucosaminyl-β(1–4)-muramic acid unit **4**. Stereoselective glycosylation of the disaccharide units was achieved by neighboring group participation of the *N*-Troc (Troc = 2,2,2-trichloroethoxycarbonyl) group and appropriate reactivity of *N*-Troc-glucosaminyl trichloroacetimidate. In a previous paper, we have reported this sequential coupling method for the synthesis of PG fragments, tetrasaccharide and octasaccharide having dipeptide units, T-2P$_2$ and O-2P$_4$.[21] The key intermediate for the synthesis of these compounds was the disaccharide **4**, which was prepared by the glycosylation of the *N*-Troc-muramyl acceptor **3** with the *N*-Troc-glucosaminyl trichloroacetimidate donor **2** in high yield (88%)[21] as shown in Scheme 1. In this reaction, the 2-*N*-Troc group was introduced instead of an acetyl group with expectation of high reactivity at the 4-hydroxy group in 2-*N*-Troc acceptors. Glycosylation of the *N*-Troc-glucosaminyl donor **2** with a *N*-Troc glucosaminyl acceptor **5** gave the disaccharide **6** in 91% yield. On the other hand, the yield of the glycosylation of *N*-Troc donor **2** with the correspoding *N*-acetyl-muramyl acceptor **7** did not exceed 50%. Recently, Crich *et al.* systematically investigated the reactivity of the 4-hydroxy group of the GlcNAc residue, which is less reactive than that of *N*,*N*-diacetyl glucosamine, *N*-acetyl-*N*-benzyl glucosamine, and 2-deoxy-2-azidoglucose derivatives owing to possible intermolecular hydrogen bond interaction.[22]

As shown in Scheme 2, disaccharide analogues were synthesized *via* the key intermediate **4**. After cleavage of the Troc group with Zn–Cu in AcOH and subsequent acetylation with Ac_2O, isomerization of the allyl group to a vinyl group was performed with H_2-activated $[Ir(cod)(MePh_2P)_2]PF_6$ to give compound **9**. The ethyl ester of **9** was cleaved with LiOH in dioxane : THF : H_2O = 2 : 4 : 1, and an appropriate peptide (see Table 1) was subsequently introduced to the liberated carboxylic acid using *N*,*N*′-diisopropylcarbodiimide (DIPC), 1-hydroxybenzotriazole (HOBt), and triethylamine in CH_2Cl_2. The cleavage of the vinyl group and the benzylidene group with 20% TFA in CH_2Cl_2, and a benzyl group at the C-terminus of peptide with hydrogenation using a $Pd(OH)_2$ catalyst gave compound **11**.



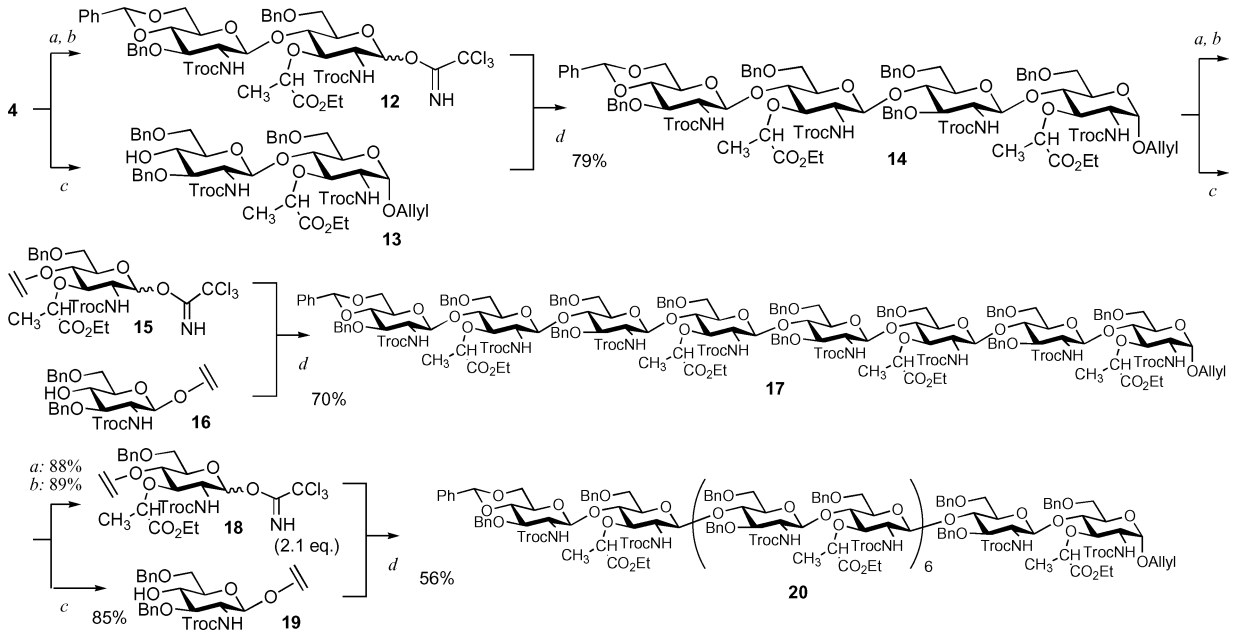
Scheme 1 Glycosylation of *N*-Troc donor with *N*-Troc acceptors.



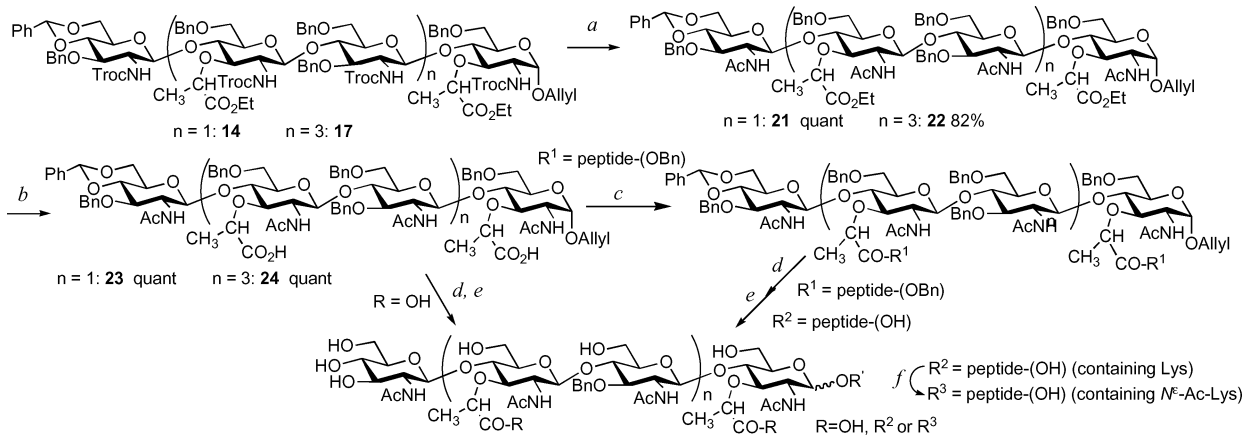
Scheme 2 Synthesis of disaccharide analogues: (a) Zn–Cu, AcOH then Ac_2O, Py., 69%; (b) Ir complex, H_2, THF, 99%; (c) LiOH, dioxane : THF : H_2O = 2 : 4 : 1, rt, quant; (d) H–R(–OBn) (R: peptide, see Table 1), DIPC, HOBt, Et_3N, CH_2Cl_2, DMF, rt, 1 d, 86%; (e) TFA (20%), CH_2Cl_2, 0 °C, 44%; (f) $Pd(OH)_2$, H_2 (20 atm), AcOH, rt, 1 d. 68%.

Tetrasaccharide **14** and octasaccharide **17** were then synthesized from **4** (Scheme 3). Deprotection of the *N*-Troc group in **14** and **17**, *N*-acetylation, saponification of ethyl esters, peptide coupling, and then final deprotection afforded tetrasaccharide with two dipeptide units **32** (T–2P$_2$) and octasaccharide with four dipeptide units **40** (O–2P$_4$), respectively (Table 1).

We also synthesized a tetrasaccharide having tri-, tetra, and pentapeptides **33** (T–3P$_2$), **35** (T–4P$_2$) and **37** (T–5P$_2$). Condensation of dicarboxylic acid **23** with appropriate peptide moieties was effected by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSCI·HCl), HOBt, and triethylamine to give protected tetrasaccharide containing two units of peptide moieties **26** (78%), **27** (50%), and **28** (68%). All benzyl and benzylidene groups of **26**, **27**, **28** were removed by catalytic hydrogenation with $Pd(OH)_2$ and H_2 to give **33** (T–3P$_2$), **35** (T–4P$_2$), **37** (T–5P$_2$), respectively. The free amino group of the Lys residue was then acetylated by treatment with Ac_2O. Purification with HP-20 column chromatography (elution with H_2O) and



Scheme 3 Synthesis of hexadecasaccharide: (a) Ir complex, H_2, THF, then I_2, H_2O; (b) CCl_3CN, Cs_2CO_3, CH_2Cl_2; (c) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, CH_3CN; (d) TMSOTf (0.1 eq), MS 4A, CH_2Cl_2, −15 °C.



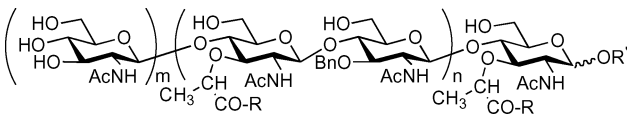
Scheme 4 Synthesis of tetrasaccharide and octasaccharide peptides: (a) Zn–Cu, AcOH then Ac_2O, Py; (b) LiOH, Dioxane, THF, H_2O; (c) appropriate peptide HCl salt, WSCI·HCl, HOBt, TEA, CH_2Cl_2; (d) Ir complex, H_2, THF, then I_2, H_2O; (e) $Pd(OH)_2$, AcOH, H_2 (10 kg cm^{-2}); (f) Ac_2O,TEA, MeOH.

gel-permeation chromatography using Sephadex LH-20 (elution with H_2O/methanol) afforded a tetrasaccharide with two peptide moieties **34** (T–3P_2A), **36** (T–4P_2A), **38** (T–5P_2A). We also synthesized glycan chains without peptide moieties. Tetrasaccharide **39** (**T**) and octasaccharide **41** (**O**) were synthesized by catalytic hydrogenolysis of **23** and **24**, respectively.

We further attempted the synthesis of hexadecasaccharide having eight dipeptide units from octasaccharide **17**. The allyl glycoside in **17** was cleaved and the product was converted to glycosyl trichloroacetimidate **18**. Regioselective-ring opening of the 4′,6′-*O*-benzylidene group in **17** with $BH_3{\cdot}Me_3N$ and $BF_3{\cdot}Et_2O$ afforded the octasaccharide acceptor **19**. Glycosylation of **19** with **18** afforded the hexadecasaccharide **20**. Unfortunately, deprotection of sixteen Troc groups in **20** by Zn–Cu in AcOH was not successful, giving a complex mixture of incompletely deprotected products having a dichloroethoxycarbonyl group. Other synthetic strategy and deprotection methods for the synthesis of the hexadecasaccharide having eight dipeptide units are now under investigation in our laboratory.

Biological activities of synthetic peptidoglycan fragments, *i.e.*, tetrasaccharide with two dipeptide units **32** (T–2P_2), octasaccharide with four dipeptide units **40** (O–2P_4), and the corresponding tetra- and octasaccharide (**T** and **O**) devoid of peptide chains, were evaluated by measuring cytokine induction from human mononuclear cells (HMC). T–2P_2 and O–2P_4 showed potent TNF-α inducing activity, whereas GlcNAc-MurNAc glycans **T**, **O** without the peptides did not show this activity (Fig. 1). Interestingly, T–2P_2 showed stronger activity than octasaccharide dipeptide O–2P_4. Both compounds were more active than natural peptidoglycan in this assay system. These results show that the peptide parts are essential for the immunostimulating activity of PG.

Table 1 Synthesized peptidoglycan fragments



m	n	*c*	Yields[a] *d*[b],*e*	*f*	Compounds	R′	R	Abbreviations
0 (H)	1	—	—	—	**1**	H	L-Ala-D-isoGln	MDP[c]
0 (H)	1	—	—	—	**30**	H	L-Ala-D-isoGln L-Lys	M–3P[c]
0 (H)	1	—	—	—	**31**	H	L-Ala-D-isoGln-L-Lys-D-Ala	M–4P[c]
1	1	—	—	—	**11**	H	L-Ala-D-isoGln-L-Lys	D–3P
1	1	**25**:86%	62%	—	**32**	H	L-Ala-D-isoGln	T–2P_2
1	1	**26**:78%	50%	—	**33**	α-Pr	L-Ala-D-isoGln-L-Lys	T–3P_2
1	1	—	—	98%	**34**	α-Pr	L-Ala-D-isoGln-L-Lys(Ac)	T–3P_2A
1	1	**27**:50%	50%	—	**35**	α-Pr	L-Ala-D-isoGln-L-Lys D-Ala	T–4P_2
1	1	—	—	85%	**36**	α-Pr	L-Ala-D-isoGln-L-Lys(Ac) D-Ala	T–4P_2A
1	1	**28**:68%	50%	—	**37**	α-Pr	L-Ala-D-isoGln-L-Lys D-Ala-D-Ala	T–5P_2
1	1	—	—	85%	**38**	α-Pr	L-Ala-D-isoGln-L-Lys(Ac) D-Ala D-Ala	T–5P_2A
1	1	—	50%	—	**39**	α-Pr	OH	T
1	3	**29**:31%	48%	—	**40**	α-Pr	L-Ala-D-isoGln	O–2P_4
1	3	—	50%	—	**41**	α-Pr	OH	O

[a] Yields in Scheme 3. [b] Deprotection of allyl group was done only for tetrasaccharide dipeptide. [c] See reference 6.

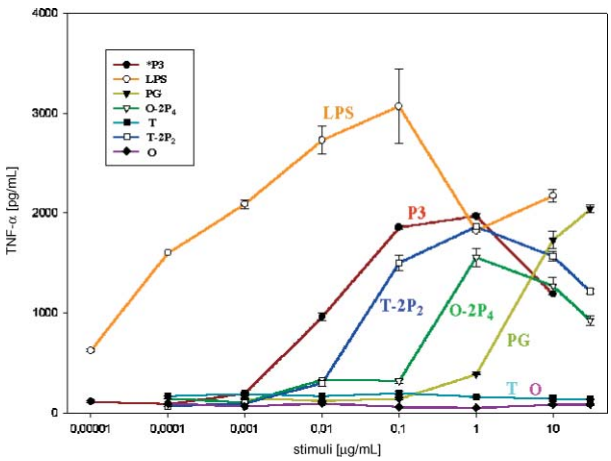


Fig. 1 TNF-α inducing activity in HMC. LPS: lipopolysaccharide, P3: synthetic lipopeptide.

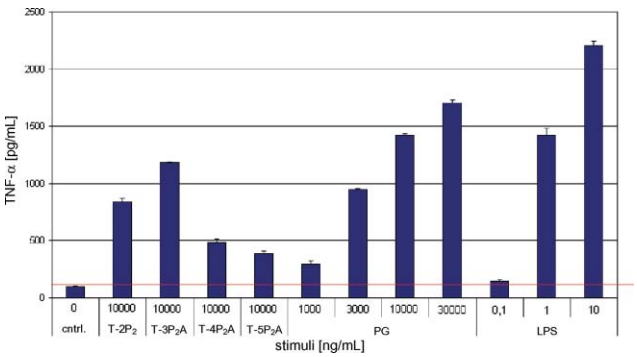


Fig. 2 TNF-α inducing activity from HMC stimulated by different synthetic PG fragments.

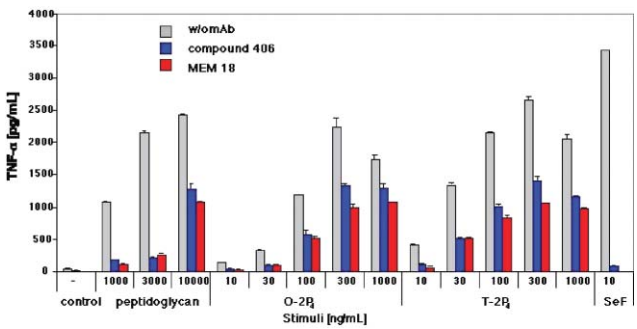


Fig. 3 CD14 dependency of immunostimulation by synthetic peptidoglycan partial structures.

Biological activities of tetrasaccharides T–3P_2A, T–4P_2A, and T–5P_2A were then compared by measuring TNF-α inducing activity from HMC. As shown in Fig. 2, tetrasaccharide including tripeptide T–3P_2A showed higher activity than the other tetrasaccharides.

We checked the CD14 dependency by using anti-CD14 monoclonal antibody (mAb) MEM-18 and compound 406,[15] as shown in Fig. 3. Compound 406 is a synthetic lipid A partial structure that shows antagonistic activity against LPS. It also inhibits the binding of PG to CD14 and consequently suppresses the activity of PG. As shown in Fig. 3, mAb MEM-18 and compound 406 partly inhibited the activation of synthetic PG partial structures T–2P_2 and O–2P_4. The present results indicate that CD14 can recognize the fundamental structure of PG.

TLR2 dependency was then checked by using anti-TLR2 antibody (Fig. 4). The TNF-α inducing activity of all synthetic PG partial structures was not inhibited by anti-TLR2, whereas the activity of PG was completely suppressed by anti-TLR2. These synthetic PG fragments were also not active in TLR2 transfected HEK293 cell (data not shown).

From the present study, tetra- and octasaccharide PG fragments were excluded from the TLR2 ligand. So far, we have clarified that partial structures having the typical peptidoglycan structure are

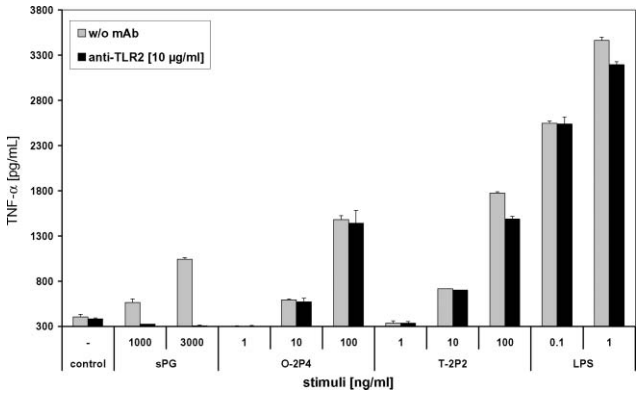


Fig. 4 Involvement of TLR2 in TNF-α inducing activity of synthetic Peptidoglycan partial structures in HMC.

not recognized by TLR2. We, however, think it is still possible that natural PGs associated with a lipid moiety would be the TLR2 ligands. Mycobacterium PG (BCG-PG) is covalently bound to mycolic acid *via* arabinogalactan. Gram-positive PG is physically associated with lipoteichoic acids. In fact, the biological activity of various 6-*O*-acylated MDP proved to be different from that of MDP.[23,24] Recently, 6-*O*-acyl-MDP derivatives were found to activate TLR2 and TLR4 on human dendritic cells as is the case with BCG-PG, although MDP expressed no ability to activate TLRs.[25] Synthetic studies of peptidoglycan partial structures are still in progress for identification of the structure that is sensed through TLR2.

Recently, the intracellular protein Nod2 was identified as a cellular receptor for MDP, and the above partial structures containing tetra- and octasaccharide T–$2P_2$ and O–$2P_4$ also showed Nod2 dependent immunostimulatory activity, whereas only glycan chains **T** and **O** did not.[26] In these PG fragments, MDP showed more potent activity than T–$2P_2$ and O–$2P_4$. We also observed the effect of a structural difference, that is the peptide length connecting the monosaccharide, with the same assay system previously reported,[26] as shown in Fig. 5. MDP also showed the most potent activity in the fragments, monosaccharide with dipeptide (MDP), tripeptide (M–3P), and tetrapeptide (M–4P). These results suggest that once the PG fragments enter a cell, Nod2 recognizes PG structures containing the MDP moiety. However, outside cells, TLR2 or other recognition proteins would recognize rather longer or lipophilic PG fragments, and this fact

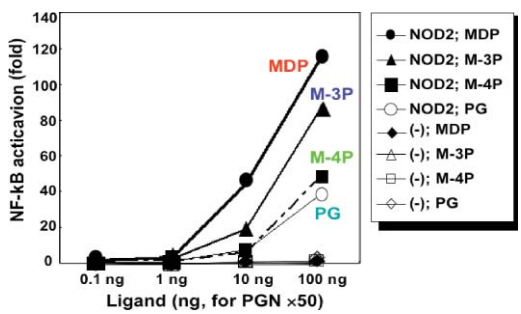


Fig. 5 Stimulation of Nod2 by PG fragments. HEK293T cells were transfected with Nod2 (Nod2) or vector control (−), and the indicated amount of each compound was added to the cells and the ability of each compound to activate NF-κB was determined.

might lead to the result that the sPG having longer glycan chains have stronger activity than MDP. As in a case of human soluble peptidoglycan recognition protein (hPGRP-S), it did not clearly bind MDP, M–3P, M–4P and T–$2P_2$ but T–$3P_2$ and T–$4P_2$, and the binding constant of T–$3P_2$ was approximately 70 times higher than that of T–$4P_2$.[27]

In conclusion, we have synthesized various PG fragments, which include monosaccharide, disaccharide, tetrasaccharide and octasaccharide with a series of peptide chains from di- to pentapeptide. For the synthesis of a longer glycan, we developed a sequencial glycosylation method and succeeded in extending the glycan chain to hexadecasaccharide. With these PG partial structures in hand, we observed the biological activity of the compounds to estimate the recognition mechanism of PG in the innate immune system. In the assay of immunostimulation activity, TLR2 did not recognize the PG fragments, but Nod2 showed recognition ability for the PG fragments containing the MDP moiety. We have also been using the PG fragments for clarifying the recognized structure of other PG recognition proteins such as hPGRP-S.[27] It was also shown that hPGRP-L recognizes M–3P as a minimal structure to hydrolyze the fragments at the linkage between muramic acid and L-Ala.[28] PG, therefore, proved to be a multifunctional immunopotentiator. Since PG and its fragments derived from natural PG may be contaminated with other immunostimulating components, the precise action mechanism of PG will be solved by using structural definite synthetic specimens.

Experimental

NMR spectra were measured with Varian INOVA-600, Varian UNITY-600, JEOL JNM-LA500, and JEOL JNM-GSX 400 spectrometers. The chemical shifts in $CDCl_3$ are given in δ values from tetramethylsilane as an internal standard. For the measurement in D_2O and CD_3OD, the HDO signal (4.66 ppm at 35 °C) and CD_3OH (4.76 ppm) were used as a reference, respectively. Mass spectra were obtained on a PerSeptive Biosystem Mariner™ Biospectrometry Workstation or a PerSeptive Biosystem Voyager Elite XL (MALDI-TOF-MS, Matrix: α-Cyano-4-hydroxycinnamic acid). Specific rotations were measured on a Perkin-Elmer 241 polarimeter. HPLC analysis was carried out with CLASS-VP system by the SHIMADZU CORPORATION. Elemental analyses were performed with a Yanaco CHN corder MT-3, MT-5 and MT-6. Silica-gel column chromatography was carried out using a Kieselgel 60 (Merck, 0.040–0.063 mm) at medium pressure (2–4 kg cm^{-2}). Dry CH_2Cl_2, THF, DMF, toluene, and benzene were purchased from Kanto Chemicals, Tokyo. Distilled water used for HPLC and lyophilization was purchased from Otsuka (Tokyo, Japan) or prepared by a combination of Tolay Pure LV-308 (Tolay) and GSL-200 (Advantec, Tokyo, Japan). Molecular Sieves (MS) 4A was activated by heating at 250 °C *in vacuo* for 3 h. Tetrakis (triphenylphosphine) Palladium (0) was prepared just before use. All other commercially obtained materials were used as received. $[Ir(cod)(MePh_2P)_2]PF_6$ activated with H_2 was used for isomerization of the 1-*O*-allyl group to the 1-*O*-(1-propenyl) group as $[Ir(cod)(H)(MePh_2P)_2]PF_6$. The activation was carried out as follows. A solution of $[Ir(cod)(MePh_2P)_2]PF_6$ in THF was degassed and then the iridium complex was activated with H_2 for 30 min. H_2 in the reaction vessel was then replaced with N_2 and

the solution was used for the isomerization. The lactic acid moiety is designated Lac in the NMR measurement.

The high resolution mass spectra of the final compounds were obtained on an Electro Spray Ionization Fourier Transform Ion Cyclotron Resonance (ESI FT-ICR) mass spectrometer, APEX II (Bruker Daltonics, Billerica, USA). The Instrument is equipped with a 7 T actively shielded magnet and an Apollo ion source. For the positive ion spectra samples ($\sim$10 ng μl^{-1}) the lyophilized substances were dissolved in a 30 : 10 (v/v) mixture of water and acetonitrile adjusted with acetic acid to pH 3.0. The samples were sprayed at a flow rate of 2 $\mu L\ min^{-1}$. The capillary entrance voltage was set to 3.8 kV, and drying gas temperature to 150 °C. For the interpretation of the plural samples, the positive or negative ion mass spectra obtained were charge deconvoluted and mass numbers refer to neutral molecules.

Allyl 6-*O*-benzyl-4-*O*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl)-3-*O*-((*R*)-1-(ethoxycarbonyl)ethyl)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside 4

To a mixture of the imidate **2** (26.0 g, 38 mmol), the acceptor **3** (17.0 g, 30 mmol), and MS4A in dry CH_2Cl_2 (300 mL) at −15 °C was added TMSOTf (340 μL, 3.0 mmol). After being stirred at the same temperature for 10 min, the reaction was quenched with chilled saturated aqueous $NaHCO_3$ (30 mL), and the mixture was extracted with $CHCl_3$ (250 mL). The organic layer was washed with $NaHCO_3$ (60 mL) and brine (60 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (600 g, toluene : EtOAc = 10 : 1) to give **4** as a pale yellow solid (29.0 g, 88%).

ESI-TOF-MS (positive) *m/z* 1119.2 $[M + Na]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.45–7.29 (15H, m, (C_6H_5)–CH_2–), 5.85–5.78 (1H, m, –CH_2–C*H*=CH_2), 5.57 (1H, s, Ph–C*H*=), 5.28–5.13 (3H, m, *H*-1, –CH_2–CH=C*H*$_2$), 4.89–4.59 (10H, m, CCl_3–C*H*$_2$–OCO–, CH_3–C*H*$_2$–OCO–, Ph–C*H*$_2$–), 4.43–4.39 (1H, m, *H*-6′), 4.26–4.04 (5H, m, Lac-α*H*, Ph–C*H*$_2$–, *H*-1′, –C*H*$_2$–CH=CH_2), 3.98–3,93 (2H, m, –CH_2–CH=C*H*$_2$, *H*-3), 3.77–3.56 (6H, m, *H*-2, *H*-4, *H*-4′, *H*-6, *H*-6′), 3.43–3.41 (2H, m, *H*-2′, *H*-5), 3.25–3.21 (2H, m, *H*-3, *H*-5′), 1.34–1.25 (6H, m, Lac-C*H*$_3$, C*H*$_3$–CH_2–OCO). Found: C, 51.42; H, 4.90; N, 2.60. Calcd for $C_{47}H_{54}Cl_6N_2O_{15}$: C, 51.33; H, 4,95; N, 2.55%.

1-Propenyl 2-acetylamino-6-*O*-benzyl-4-*O* (2-acetylamino-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl-3-*O*-((*R*)-1-(ethoxycarbonyl)ethyl)-2-deoxy-α-D-glucopyranoside 9

To a solution of **4** (2.1 g, 1.9 mmol) in AcOH (2.5 mL) was added Zn–Cu (prepared from 5.6 g of Zn) and the mixture was stirred at room temperature for 4 h. The insoluble materials were filtered off and the filtrate was concentrated *in vacuo*. The residual solvent was removed by coevaporation with toluene (10 mL) The residue was dissolved in pyridine (40 mL) and acetic anhydride (42 mL) and the solution was stirred at room temperature. After 1 day, the solution was removed by concentration with toluene (10 mL). The residue was purified by silica-gel flash chromatography (150 g, $CHCl_3$: acetone = 7 : 1) to give 2,2′-*N*,*N*-diacetyl-disaccharide as a white solid (1.1 g, 69%). ESI-TOF-MS (positive) *m/z* 832.4 $[M + Na]^+$;^{1}H-NMR 500 MHz, $CDCl_3$ δ = 7.53–7.15(m, 15 H, C_6H_5–CH_2–), 5.87–5.82(m, 1H, –CH_2–C*H*=CH_2), 5.58(s, 1H, C_6H_5–C*H*–), 5.31–5.13 (m, 3H, H1, –CH_2–CH=C*H*$_2$), 4.89–4.82(m, 2H, C_6H_5–C*H*$_2$–), 4.66–4.60 (m, 2H, CH_3–C*H*$_2$–O–CO–), 4.45–4.34(m, 3H, C_6H_5–C*H*$_2$–, H6′), 4.26–4.12(m, 2H, H1′, Lac-αH), 4.08–4.06(m, 2H, –C*H*$_2$–CH=CH_2), 3.96–3.92 (m, 2H, –C*H*$_2$–CH=CH_2, H3), 3.78–3.44 (m, 9H, H2, H4, H6, H3′, H4′, H6′), 3.32–3.29 (m, 1H, H5′), 2.01 (s, 3H, –NH–CO–C*H*$_3$), 1.74 (s, 3H, –NH–CO–C*H*$_3$), 1.74–1.26 (m, 6H, Lac-CH_3, C*H*$_3$–CH_2–O–CO–).

To a solution of the 2,2′-*N*,*N*-diacetyl-disaccharide (100 mg, 0.12 mmol) in dry THF (2.5 mL) was added H_2-activated $[Ir(cod)(MePh_2P)_2]PF_6$ (3.1 mg, 3.6×10^{-3} mmol) in dry THF (1 mL). After being stirred under an argon atmosphere at room temperature for 7 h, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ (20 mL) and the mixture was extracted with AcOEt (30 mL × 2). The organic layer was washed with sat. $NaHCO_3$ aq (30 mL) and brine (30 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (5 g, $CHCl_3$: acetone = 7 : 1) to give **9** as a white solid (100 mg, 99%). ESI-TOF-MS (positive) *m/z* 832.4 $[M + Na]^+$;^{1}H-NMR 500 MHz, $CDCl_3$ δ = 7.53–7.14(m, 15 H, C_6H_5–CH_2–), 6.10 (d, J = 12.2 Hz, 1H, –C*H*=CH–CH_3), 5.59(s, 1H, C_6H_5–C*H*–), 5.51 (d, J = 1.0 Hz, 1H, H1), 5.08–5.05(m, 1H, –CH=C*H*–CH_3), 4.89–4.82(dd, J = 12.6 Hz, 2H, C_6H_5–C*H*$_2$–), 4.66–4.62 (m, 2H, CH_3–C*H*$_2$–O–CO–), 4.44–4.32(m, 3H, C_6H_5–C*H*$_2$-, H6′), 4.24–4.15(m,2H, H1′, Lac-αH), 3.96 (t, J = 9.1 Hz, 1H, H3), 3.78–3.43 (m,9H, H2, H4, H5,H6, H2′, H3′, H4′, H6′), 3.30–3.29 (m,1H, H5′), 2.00 (s, 3H, –NH–CO–C*H*$_3$), 1.52 (d, J = 6.9 Hz, 3H, –CH=CH–C*H*$_3$), 1.73 (s, 3H, –NH–CO–C*H*$_3$), 1.37–1.26 (m, 6H, Lac-CH_3, C*H*$_3$–CH_2–O–CO–).

Protected disaccharide tripeptide 10: (R=L-Ala-D-isoGln-L-Lys(Z)–OBn)

To a solution of **9** (110 mg, 0.13 mmol) in dioxane : THF : H_2O (2 : 4 : 1, 4.0 mL) was added LiOH (19 mg, 0.79 mmol) and stirred at room temperature for 1 h. The solution was neutralized with Dowex H^+ (Dowex 50W × 8 200–400 mesh H form, DowChemicals) and then applied to an HP-20 column (2 cm × 20 cm). Organic and inorganic salts were removed by elution with H_2O (160 mL), then eluted with MeOH and concentrated *in vacuo* to give a disaccharide with a free lactic acid moiety as a white solid (105 mg, quant.). ESI-TOF-MS (positive) *m/z* 805.4 $[M + H]^+$;^{1}H-NMR (500 MHz, $CDCl_3$) δ = 7.48–7.23(m, 15 H, C_6H_5–CH_2–), 6.13 (d, J = 10.5 Hz, 1H, –C*H*=CH–CH_3), 5.64(s, 1H, C_6H_5–C*H*–), 5.43 (d, J = 3.1 Hz, 1H, H1), 5.06–5.02(m, 1H, –CH=C*H*–CH_3), 4.87–4.79(m, 2H, C_6H_5–C*H*$_2$–), 4.64–4.56 (m, 3H, C_6H_5–C*H*$_2$-, H6′, Lac-αH), 4.31–4.28(m, 1H, H1′), 4.07–3.49 (m, 11H, H2, H3, H4, H5,H6, H2′, H3′, H4′, H6′), 3.30–3.29 (m, 1H, H5′), 1.98 (s, 3H, –NH–CO–C*H*$_3$), 1.87 (s, 3H, –NH-CO-C*H*$_3$), 1.50 (d, J = 1.5 Hz, 3H, –CH=CH-C*H*$_3$), 1.38 (d, J = 6.9 Hz, 3H, Lac-CH_3).

To a solution of the above disaccharide (105 mg, 0.13 mmol) in dry DMF : CH_2Cl_2 (1 : 1, 4.0 mL) was added HOBt (24 mg, 0.18 mmol) and DIPC (2.8×10^{-2} mL, 0.18 mmol) and stirred under an argon atmosphere at room temperature for 1 h. To a solution of HCl·H-L-Ala-D-isoGln-L-Lys(Z)–OBn (71 mg, 0.12 mmol) in dry DMF : CH_2Cl_2 (1 : 1, 4.0 mL) was added Et_3N (1.6×10^{-2} mL, 0.12 mmol) and the above mixture, and the reaction mixture was stirred under an argon atmosphere at room

temperature overnight. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in $CHCl_3$ (50 mL). The $CHCl_3$ solution was washed with 10% aqueous citric acid (50 mL), saturated aqueous $NaHCO_3$ (80 mL) and brine (80 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (50 g, $CHCl_3$: MeOH = 10 : 1) to give **10** as a white solid (138 mg, 87%). ESI-TOF-MS (positive) *m/z* 1378.7 $[M + Na]^+$;^{1}H-NMR (500 MHz, $CDCl_3$) δ = 7.48–7.23(m, 25 H, C_6H_5–CH_2–), 6.13 (d, J = 12.0 Hz, 1H, –C*H*=CH–CH_3), 5.61(s, 1H, C_6H_5–C*H*–), 5.36 (d, J = 3.5 Hz, 1H, H1), 5.22–5.09(m, 6H, –CH=C*H*–CH_3, C_6H_5–CH_2–, C_6H_5–CH_2–OCO–), 4.89–4.87(d, J = 11.5 Hz, 1H, C_6H_5–CH_2–), 4.73–4.61 (m, 3H, C_6H_5–CH_2–), 4.54–4.23 (m, 5H, H6′, Lac-αH, Lys-αH, Gln-αH, Ala-αH), 4.23–3.93(m, 2H, H1′, H3), 3.84–3.56(m,8H, H2, H4, H5,H6, H2′, H4′, H6′), 3.40–3.31 (m, 2H, H3′, H5′), 3.18–3.07 (m,2H, Lys-εCH_2), 2.42–2.28 (m, 2H, Gln-γCH_2), 2.25–2.20 (m, 2H, Gln-βCH_2), 1.97 (s, 3H, –NH–CO–CH_3), 1.90 (s, 3H, –NH–CO–CH_3), 1.80–1.57 (m, 4H, Lys-βCH_2, Lys-δ CH_2), 1.55–1.35 (m, 4H, Lys-γCH_2, Ala-CH_3), 1.28–1.12 (m, 6H, –CH=CH–CH_3, Lac-CH_3).

Disaccharide tripeptide 11

A solution of **10** (2.8 mg, 2.1 × 10^{-3} mmol) in TFA : CH_2Cl_2 (1 : 4, 0.5 mL) was stirred at 0 °C for 1.5 h. The reaction mixture was quenched with saturated aqueous $NaHCO_3$ (20 mL) and the mixture was extracted with AcOEt (50 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ (20 mL) and dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (5 g, $CHCl_3$: MeOH = 8 : 1) to give 1-liberated disaccharide as a white solid (1.1 mg, 44%). ESI-TOF-MS (positive) *m/z* 1250.7 $[M + Na]^+$;^{1}H-NMR (500 MHz, $CDCl_3$) δ = 7.28–7.17(m, 25 H, C_6H_5–CH_2–), 5.17(d, J = 3.5 Hz, 1H, H1), 5.07–4.81(m, 4H, C_6H_5–CH_2–, C_6H_5–CH_2–OCO–), 4.56–4.42 (m, 8H, C_6H_5–CH_2–, H6′), 4.30–4.18 (m, 5H, Lac-αH, Lys-αH, Gln-αH, Ala-αH, H1′), 3.97–3.95(m, 1H, H3), 3.80–3.32(m,12H, H2, H4, H5, H6, H2′, H3′, H4′, H5′, H6′,), 3.12–2.96 (t, J = 6.8 Hz, 2H, Lys-εCH_2), 2.26–2.22 (m, 2H, Gln-γCH_2), 2.10–2.06 (m, 2H, Gln-βCH_2), 1.85 (s, 3H, –NH–CO–CH_3), 1.78 (s, 3H, –NH–CO–CH_3), 1.73–1.50 (m, 4H, Lys-βCH_2, Lys-δCH_2), 1.37–1.33 (m, 8H, Lys-γCH_2, Ala-CH_3, Lac-CH_3).

To a solution of the 1-librarated disaccharide (1.1 mg, 9.0 × 10^{-4} mmol) in acetic acid (2.0 mL) was added palladium hydroxide (2.0 mg, 1.4 × 10^{-2} mmol) in acetic acid (1.0 mL), and the mixture was stirred under H_2 (20 atm) at room temperature overnight. The Pd catalyst was removed by filtration and the filtrate was concentrated *in vacuo* to give **11** as a white solid (0.5 mg, 68%). ESI-TOF-MS (positive) *m/z* 824.4 $[M + Na]^+$. HRMS-ESI FT-ICR (positive): (M) calcd for $C_{33}H_{57}N_7O_{17}$, 823.381; found, 823.393.

Disaccharide 1-*O*-trichloroacetimidate 12

To a degassed solution of **4** (3.0 g, 2.7 mmol) in dry THF (6 mL) was added $[Ir(cod)(MePh_2P)_2]PF_6$ (23 mg, 0.027 mmol) activated with H_2. After being stirred under a nitrogen atmosphere at room temperature for 1 h, the solution of $[Ir(cod)(MePh_2P)_2]PF_6$ (23 mg, 0.027 mmol) activated with H_2 in dry THF (3 mL) was added. After being stirred under a nitrogen atmosphere at room temperature for 1 h, iodine (690 mg, 2.7 mmol) and water (10 mL) were added and the reaction mixture was stirred for additional 10 min. To the reaction mixture was rapidly added aqueous $Na_2S_2O_3$ (5%, 100 ml). The mixture was then extracted with EtOAc (50 mL). The organic layer was washed with aqueous $Na_2S_2O_3$ (5%, 50 mL × 2), aqueous sat. $NaHCO_3$ (100 mL × 2), brine (50 mL), dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (180 g, toluene : EtOAc = 4 : 1) to give 1-liberated-disaccharide as a pale yellow solid (2.72 g, 93%).

$[\alpha]_D^{23}$ = + 8.4 (*c* 1.00, $CHCl_3$); ESI-MS (positive) *m/z* = 1079.0 $[M + Na]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.54–7.27 (m, 15H, (C_6H_5)–CH_2–), 5.60 (br.s, 1H, H-1), 5.57 (s, 1H, Ph–C*H*=), 4.89–4.60 (m, 8H, CCl_3–CH_2–OCO–, CH_3–CH_2–OCO–, Ph–CH_2–), 4.43–4.39 (m, 1H, H-6‴), 4.31–3.91 (m, 4H, Ph–CH_2–, H-1′, Lac-αH), 3.95–3.91 (m, 1H, *H-3*), 3.82–3.63 (m, 6H, *H-2*, *H-4*, *H-6*, *H-4′*, *H-6′*), 3.43–3.41 (m, 2H, *H-2′*, *H-5*), 3.22–3.21 (m, 2H, *H-3′*, *H-5′*), 1.35–1.27 (m, 6H, Lac-*Me*, C*H*$_3$–CH_2–OCO). Found: C, 51.68; H, 5.33; N, 2.35. Calcd for $C_{44}H_{50}C_{16}N_2O_{15}$: C, 51.24; H, 5.12; N, 2.54%.

To a solution of 1-liberated-disaccharide (2.72 g, 2.56 mmol) in dry CH_2Cl_2 (6 mL) at rt were added Cs_2CO_3 (417 mg, 1.28 mmol) and CCl_3CN (3.7 mL, 25.6 mmol). After being stirred for 1 h, insoluble materials were filtered off through celite and concentrated. The residue was lyophilized from benzene to give **12** as a pale yellow solid (3.04 g), which was used for subsequent glycosylation without purification.

4′-*O*-Deprotected-disaccharide 13

To a solution of **4** (1.5 g, 1.36 mmol) and trimethylamine-borane (150 mg, 2.05 mmol) in dry CH_3CN (13 mL) at 0 °C was added boron trifluoride diethyl etherate (960 mg, 6.80 mmol) dropwise and the mixture was stirred at rt for 30 min. The reaction was then quenched with ice and saturated aqueous $NaHCO_3$ (100 mL) and the mixture was extracted with EtOAc (100 mL × 2). The organic layer was washed with aqueous 10% citric acid (15 mL × 4), saturated aqueous $NaHCO_3$ (150 mL), and brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (180 g, toluene : AcOEt = 4 : 1) to give **13** as a colorless solid (1.13 g, 73%).

$[\alpha]_D^{23}$ = + 25.6 (*c* 1.00, $CHCl_3$); ESI-TOF-MS (positive) *m/z* = 1121.6 $[M + Na]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.43–7.27 (15H, m, (C_6H_5)–CH_2–), 5.85–5.79 (1H, m, –CH_2–C*H*=CH_2), 5.26–5.13 (3H, m, –CH_2–CH=CH_2, H-1), 4.86–4.50 (9H, m, CCl_3–CH_2–OCO–, CH_3–CH_2–OCO–, Ph–CH_2–), 4.33–4.04 (5H, m, Ph–CH_2–, Lac-α*H*, *H*-1′, –CH_2–CH=CH_2), 3.97–3.57 (10H, m, –CH_2–CH=CH_2, *H*-3, *H*-4, *H*-6, *H*-3′, *H*-4′, *H*-6′), 3.46–3.36 (2H, m, *H*-2′, *H*-5), 3.27–3.23 (1H, m, *H*-5′), 1.29–1,20 (6H, m, Lac-C*H*$_3$, C*H*$_3$–CH_2–OCO). Found: C, 51.68; H, 5.33; N, 2.35. Calcd for $C_{47}H_{56}C_{16}N_2O_{15}$: C, 51.24; H, 5.12; N, 2.54%.

Fully protected tetrasaccharide 14

To a mixture of the imidate **12** (2.7 g, 38 mmol), the acceptor **13** (1.65 g, 30 mmol), and MS4A in dry CH_2Cl_2 (75 mL) at −15 °C was added TMSOTf (18 μL, 0.15 mmol). After being stirred at the same temperature for 10 min, the reaction was quenched with ice cold saturated aqueous $NaHCO_3$ (100 mL), and the mixture was extracted with $CHCl_3$ (100 mL). The organic layer was washed

with $NaHCO_3$ (60 mL × 2) and brine (60 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (300 g, toluene : EtOAc = 6 : 1) to give **14** as a pale yellow solid (2.33 g, 79%).

$[\alpha]_D^{23} = -1.7$ (*c* 1.00, $CHCl_3$); ESI-TOF-MS (positive) *m/z* = 2159.0 $[M + Na]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ = 7.51–7.27 (30H, m, (C_6H_5)–CH_2–), 5.86–5.76 (1H, m, –CH_2–CH=CH_2), 5.56 (1H, s, Ph–CH=), 5.25–4.98 (4H, m, –CH_2–CH=CH_2, Ph–CH_2–, *H*-1), 4.88–4.33 (17H, m, CCl_3–CH_2–OCO–, CH_3–CH_2–OCO–, Ph–CH_2–), 4.32–3.90 (12H, m, Ph–CH_2–, Lac-αH, *H*-1″, *H*-1″, *H*-1‴, *H*-6‴, –CH_2–CH=CH_2), 3.87–3.01 (24H, m, Ph–CH_2–, *H*-2, *H*-3, *H*-4, *H*-5, *H*-6, *H*-2′, *H*-3′, *H*-4′, *H*-5′, *H*-6′, *H*-2″, *H*-3″, *H*-4″, *H*-5″, *H*-6″, *H*-2‴, *H*-3‴, *H*-4‴, *H*-5‴, *H*-6‴), 1.32–1,27 (12H, m, Lac-CH_3, CH_3–CH_2–OCO).

Tetrasaccharide 1-*O*-trichloroacetimidate 15

To a degassed solution of **14** (5.4 g, 2.5 mmol) in dry THF (50 mL) was added H_2 activated (30 min) $[Ir(cod)(MePh_2P)_2]PF_6$ (100 mg, 0.12 mmol) in dry THF (3 mL). After being stirred under a nitrogen atmosphere at room temperature for 1 h, iodine (1.26 g, 5.0 mmol) and water (10 mL) were added and the reaction mixture was stirred for additional 10 min. To the reaction mixture was added rapidly aqueous $Na_2S_2O_3$ (5%, 150 ml). The mixture was then extracted with EtOAc (100 mL). The organic layer was washed with aqueous $Na_2S_2O_3$ (5%, 100 mL × 2), aqueous sat. $NaHCO_3$ (100 mL × 2) and brine (50 mL) then dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (300 g, toluene : EtOAc = 4 : 1) to give 1-liberated tetrasaccharide as a pale yellow solid (4.3 g, 81%).

$[\alpha]_D^{23} = -11.0$ (*c* 1.00, $CHCl_3$); ESI-TOF-MS (positive) *m/z* = 2119.7 $[M + Na]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ = 7.62–7.27 (30H, m, (C_6H_5)–CH_2–), 5.62 (1H, br.s, *H*-1), 5.56 (1H, s, Ph–CH=), 5.11 (1H, d, *J* = 12,2 Hz, Ph–CH_2–), 4.87–4.46 (19H, m, CCl_3–CH_2–OCO–, CH_3–CH_2–OCO–, Ph–CH_2–, *H*-1″, *H*-6‴), 4.31–3.12 (7H, m, Ph–CH_2–, *H*-1′, *H*-1‴, Lac-αH), 3.96–3.44 (19H, m, Ph–CH_2–, *H*-2, *H*-3, *H*-4, *H*-5, *H*-6, *H*-2′, *H*-4′, *H*-6′, *H*-3″, *H*-4″, *H*-5″, *H*-6″, *H*-2‴, *H*-4‴, *H*-6‴), 3.36–3.18 (5H, m, *H*-3′, *H*-5′, *H*-2″, *H*-3‴, *H*-5‴). 1.36–1,25 (12H, m, Lac-CH_3, CH_3–CH_2–OCO).

To a solution of 1-liberated tetrasaccharide (4.3 g, 2.0 mmol) in dry CH_2Cl_2 (20 mL) at rt were added Cs_2CO_3 (325 mg, 1.0 mmol) and CCl_3CN (2.0 mL, 20.0 mmol). After being stirred for 1 h, insoluble materials were filtered off through celite and the filtrate was concentrated. The residue was lyophilized from benzene to give **15** as a pale yellow solid (3.8 g), which was used for subsequent glycosylation without purification.

4‴-*O*-Deprotected-tetrasaccharide 16

To a solution of **14** (1.9 g, 0.89 mmol) and trimethylamine-borane (128 mg, 1.77 mmol) in dry CH_3CN (36 mL) at rt was added boron trifluoride diethyl etherate (461 μL, 3.56 mmol) diluted with CH_3CN (1.4 mL) dropwise and the mixture was stirred at rt for 1 h. The reaction was then quenched with saturated aqueous $NaHCO_3$ (50 mL) and the mixture was extracted with EtOAc (100 mL). The organic layer was washed with aqueous 10% citric acid (150 mL × 4), saturated aqueous $NaHCO_3$ (150 mL), and brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (200 g, toluene : AcOEt = 4 : 1) to give **16** as a colorless solid (1.4 g, 74%).

$[\alpha]_D^{23} = -24.3$ (*c* 1.00, $CHCl_3$); ESI-TOF-MS (positive) *m/z* 2161.4 $[M + Na]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ = 7.43–7.28 (30H, m, (C_6H_5)–CH_2–), 5.86–5.80 (1H, m, –CH_2–CH=CH_2), 5.28–5.11 (1H, m, –CH_2–CH=CH_2), 5.57 (1H, d, *J* = 12,2 Hz, Ph–CH_2–), 4.88–4.50 (19H, m, CCl_3–CH_2–OCO–, CH_3–CH_2–OCO–, Ph–CH_2–), 4.45–3.86 (14H, m, Ph–CH_2–, CCl_3–CH_2–OCO–, *H*-1, *H*-1′, *H*-1″, *H*-1‴, Lac-αH, –CH_2–CH=CH_2), 3.83–2.84 (24H, m, *H*-2, *H*-3, *H*-4, *H*-5, *H*-6, for each four pyranose), 1.31–1,24 (12H, m, Lac-Me, CH_3–CH_2–OCO).

Fully-protected octasachharide 17

To a mixture of the imidate **15** (3.8 g, 1.7 mmol), the acceptor **16** (2.4 g, 1.1 mmol), and MS4A in dry CH_2Cl_2 (112 mL) at −15 °C was added TMSOTf (13 μL, 0.11 mmol). After being stirred at the same temperature for 10 min, the reaction was quenched with chilled saturated aqueous $NaHCO_3$ (100 mL), and $CHCl_3$ (50 mL) was added. The organic layer was washed with $NaHCO_3$ (60 mL × 2) and brine (60 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (300 g, toluene : EtOAc = 5 : 1) to give **17** as a pale yellow solid (3.2 g, 70%).

$[\alpha]_D^{23} = -20.2$ (*c* 1.00, $CHCl_3$); MALDI-TOF-MS (positive) *m/z* = 4253.42 $[M + Na]^+$; Found: C, 50.68; H, 4.96; N, 2.70. Calcd for $C_{179}H_{204}Cl_{24}N_8O_{57}\cdot 1H_2O$: C, 50.60; H, 4.81; N, 2.63%.

Octasaccharide 1-*O*-trichloroacetoimidate 18

To a degassed solution of **17** (300 mg, 0.071 mmol) in dry THF (2 mL) was added $[Ir(cod)(MePh_2P)_2]PF_6$ (6 mg, 0.007 mmol) activated with H_2 in THF (3 mL). After being stirred under nitrogen atmosphere at room temperature for 1 h, iodine (35 mg, 0.142 mmol) and water (0.5 mL) were added and the reaction mixture was stirred for additional 10 min. To the reaction mixture was added rapidly aqueous $Na_2S_2O_3$ (5%, 10 ml). The mixture was then extracted with EtOAc (20 mL). The organic layer was washed with aqueous $Na_2S_2O_3$ (5%, 10 mL × 2), aqueous sat. $NaHCO_3$ (50 mL × 2), and brine (20 mL, dried over Na_2SO_4, and then concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (20 g, toluene : EtOAc = 5 : 1) to give 1-liberated-octasaccharide as a pale yellow solid (260 mg, 88%).

MALDI-TOF-MS (positive) *m/z* = 4213.8 $[M + Na]^+$.

To a solution of 1-liberated-octasaccharide (260 mg, 0.062 mmol) in dry CH_2Cl_2 (1.2 mL) at rt were added Cs_2CO_3 (4 mg, 0.62 mmol) and CCl_3CN (62 μL, 0.62 mmol). After being stirred for 1 h, insoluble materials were filtered off through celite and the filtrate was concentrated. The residue was lyophilized from benzene to give **18** as a pale yellow solid (240 mg).

4-*O*-Deprotected octasaccharide 19

To a solution of **17** (100 mg, 0.023 mmol) and trimethylamine-borane (5.4 mg, 0.026 mmol) in dry CH_3CN (4.8 mL) at 0 °C was added boron trifluoride diethyl etherate (9.6 μL, 0.071 mmol) in dry CH_3CN (28 μL) dropwise and the mixture was stirred at rt for 1 h. The reaction was then quenched with saturated aqueous $NaHCO_3$ (5 mL) and the mixture was extracted with EtOAc (10 mL). The organic layer was washed with aqueous 10%

citric acid (15 mL × 4), saturated aqueous $NaHCO_3$ (15 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (10 g, toluene : AcOEt = 4 : 1) to give **19** as a colorless solid (85 mg, 85%).

MALDI-TOF-MS (positive) $m/z = 4255.00$ $[M + Na]^+$.

Hexadecasaccharide 20

To a mixture of the imidate **18** (85 mg, 0.02 mmol), the acceptor **19** (240 mg, 0.05 mmol), and MS4A in dry CH_2Cl_2 (3 mL) at −15 °C was added TMSOTf (0.4 mg, 2.0 μmol). After being stirred at the same temperature for 10 min, the reaction was quenched with ice cold saturated aqueous $NaHCO_3$ (5 mL), and the mixture was added with $CHCl_3$ (5 mL). The organic layer was washed with $NaHCO_3$ (5 mL × 2) and brine (5 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (80 g, toluene : EtOAc = 4 : 1) to give **20** as a pale yellow solid (87 mg, 56%).

MALDI-TOF-MS (positive) m/z 8427.3 $[M + Na]^+$

2-*N*-Acetyl-tetrasaccharide 21

To a solution of **14** (1.05 g, 0.46 mmol) in AcOH (10 mL) was added Zn–Cu (prepared from 1 g of Zn), and the mixture was stirred at rt for 1 h. The insoluble materials were filtered off and the filtrate was concentrated *in vacuo*. The residual solvent was removed by coevaporation with toluene (5 mL × 3). The crude product was dissolved in pyridine (7 mL) and acetic anhydrate (7 mL) and the solution was stirred at rt. After 30 min, the solution was removed by concentration *in vacuo*. The residual solvent was removed by coevaporation with toluene (5 mL × 3). The residue was purified by silica-gel flash chromatography (80 g, CH_3Cl : acetone = 3 : 1) to give **21** as a white solid (750 mg, quant.).

$[\alpha]_D^{23} = -7.4$ (*c* 1.00, $CHCl_3$); ESI-TOF-MS (positive) $m/z =$ 1609.2 $[M + H]^+$, 1631.6 $[M + Na]^+$.

2-*N*-Acetyl-octasacchride 22

To a solution of **17** (1.0 g, 0.23 mmol) in AcOH (15 mL) was added Zn–Cu (prepared from 2 g of Zn), and the mixture was stirred at rt for 1 h. The insoluble materials were filtered off and the filtrate was concentrated *in vacuo*. The residual solvent was removed by coevaporation with toluene (5 mL × 3). The crude product was dissolved in pyridine (15 mL) and acetic anhydrate (16 mL) and the solution was stirred at rt. After 30 min, the solution was concentrated *in vacuo*. The residual solvent was removed by coevaporation with toluene (5 mL x3). The residue was purified by silica-gel flash chromatography (80 g, CH_3Cl : acetone = 3 : 1) to give **22** as a white solid (600 mg, 83%).

ESI-TOF-MS (positive) $m/z = 3183.71$ $[M + Na]^+$.

Tetrasaccharide with librareted carboxylic acid 23

To a solution of **21** (180 mg, 0.11 mmol) in dioxane : THF : H_2O (2 : 4 : 1, 1.2 mL) was added LiOH (28 mg, 0.66 mmol) and stirred at rt for 1 h. The solution was neutralized with Dowex H^+(Dowex 50 W × 8 200–400 mesh H form, DowChemicals) and then applied to an HP-20 column (2 cm × 40 cm). Organic and inorganic salts were removed by elution with H_2O (300 mL), then eluted with MeOH to give **23** as a white solid (170 mg, quant.).

ESI-TOF-MS (positive) $m/z = 1575.1$ $[M + Na]^+$.

Octasaccharide with liberated carboxylic acid 24

To a solution of **22** (28 mg, 9.0 μmol) in dioxane : THF : H_2O (2 : 4 : 1, 4 mL) was added LiOH (3 mg, 0.073 mmol) and stirred at rt for 1 h. The solution was neutralized with Dowex H^+(Dowex 50W-X8 200–400 mesh H form, DowChemicals) and then applied to HP-20 column (2 cm × 40 cm). Organic and inorganic salts were removed by elution with H_2O (300 mL), then eluted with MeOH to give **24** as a white solid (28 mg, quant.).

MALDI-TOF-MS (positive) $m/z = 3115.26$ $[M + Na]^+$.

Protected tetrasaccharide dipeptide 25

To a solution of **23** (122 mg, 0.078 mmol, HCl·H-L-Ala-D-Glu(OBn)–NH_2 (83 mg, 0.24 mmol), and HOBt (33.5 mg, 0.25 mmol) in CH_2Cl_2(14 mL) were added WSCI·HCl (37 mg, 0.25 mmol) and triethylamine (48 μL, 0.47 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was diluted with AcOEt and insoluble materials were filtered off. The filtrate was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O(20 mL), saturated aqueous $NaHCO_3$ (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (20 g, $CHCl_3$: MeOH = 20 : 1) to give **25** as a white solid (143 mg, 86%).

ESI-TOF-MS (positive) $m/z = 2153.52$ $[M + Na]^+$;^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 7.52$–7.15 (40H, m), 5.58 (1H, m), 5.57 (1H, s), 5.56–5.07 (6H, m), 4.86 (1H, d, $J = 12.3$ Hz), 4.83 (1H, d, $J = 3.7$ Hz), 4.74 (1H. dd, $J = 12.1$ Hz), 4.66–4.57 (4H, m), 4.35–4.24 (8H, m), 4.09–3.92 (6H, m), 3.83–3.59 (12H, m), 3.53–3.43 (10H, m), 3.39–3.35 (1H, m), 3.34–3.20 (1H, m), 2.56–2.41 (4H, m), 2.17–2.03 (7H, m), 1.93 (3H, s), 1.88 (3H, s), 1.73 (3H, s), 1.57–1.53 (3H, m), 1.43 (1H, d, $J = 6.9$ Hz), 1.37–1.33 (3H, m), 1.26 (1H, m). Found: C, 62.03; H, 6.63; N, 6.38. Calcd for $C_{113}H_{138}N_{10}O_{31}\cdot 3H_20$: C, 62.08; H, 6.64; N, 6.41%.

Proteced tetrasaccharide tripeptide 26. ESI-TOF-MS (positive) $m/z = 1330.49$ $[M + 2H]^{2+}$, tetrapeptide **27** ESI-TOF-MS (positive) $m/z = 1400.53$ $[M + 2H]^{2+}$, pentapeptide **28** ESI-TOF-MS (positive) $m/z = 1471.47$ $[M + 2H^{2+}]$, octasaccharide dipeptide **29** ESI-TOF-MS (positive) $m/z = 4228.27$ $[M + Na]^+$ were synthesized in a manner similar to **25**.

Tetrasaccharide 39. To a solution of **23** (50 mg, 0.032 mmol) in acetic acid (3 mL) was added palladium hydroxide on carbon (100 mg) in acetic acid(1 mL) and the mixture was stirred under H_2 (10 atm) for one day. The Pd catalyst was removed by filtration and the filtrate was concentrated. The residue was purified with LH-20 column by elution with MeOH to give **39** as a white solid (7.3 mg, 22%). MALDI-TOF-MS (negative) $m/z = 1015.9$ $[M - H]^-$.

Tetrasaccharide dipeptide 32. To a degassed solution of **25** (300 mg, 0.071 mmol) in dry THF (2 mL) was $[Ir(cod)(MePh_2P)_2]PF_6$ (6 mg, 0.007 mmol) activated with H_2 in dry THF (3 mL). The solution was stirred under a nitrogen atmosphere at rt for 1 h, iodine (35 mg, 0.142 mmol) and water

(0.5 mL) were added and the reaction mixture was stirred for additional 10 min. The reaction was quenched by the addition of aqueous $Na_2S_2O_3$ (5%, 10 ml). The mixture was then extracted with EtOAc (20 mL). The organic layer was washed with aqueous $Na_2S_2O_3$ (5%, 10 mL × 2), aqueous sat. $NaHCO_3$ (50 mL × 2), and brine (20 mL), dried over Na_2SO_4, and then concentrated *in vacuo*. The residue was purified by silica-gel flash chromatography (20 g, toluene : EtOAc = 5 : 1) to give 1-liberated-tetrasaccharide as a pale yellow solid (260 mg, 88%). ESI-TOF-MS (positive) m/z = 2113.6 $[M + Na]^+$.

To a solution of the above 1-liberated-tetrasaccharide (86 mg, 0.04 mmol) in acetic acid (3 mL) was added palladium hydroxide on carbon (100 mg) in acetic acid(1 mL) and the mixture was stirred under H_2 (20 atm) for one day. The Pd catalyst was removed by filtration and the filtrate was concentrated and lyophilized from H_2O to give **32** (T–2P_2) as a white solid (39 mg, 70%). ESI-TOF-MS (negative) m/z = 685.3 $[M - 2H]^{2-}$; HRMS-ESI FT-ICR (negative): (M) calcd for $C_{54}H_{88}N_{10}O_{31}$, 1372.561; found, 1372.555; ^{1}H NMR (600 MHz, D_2O): δ = 5.16–5.15 (d, J = 3.0 Hz, 1H, H-1), 4.46–4.42 (m, 3H), 4.36–4.32 (m, 2H), 4.30–4.27 (m, 2H, iGln-α-C*H*), 4.26–4.19 (m, 2H), 3.86–3.30 (m, 24H), 2.31 (m, 4H, iGln-γ-CH_2), 2.12–2.03 (m, 4H, iGln-β-CH_2), 1.96–1.95 (s, 12H, NHC(O)CH_3 × 4), 1.37–1.35 (m, 6H, Ala-β-CH_3), 1.31–1.28 (m, 6H, Lac-β-CH_3).

Tetrasaccharide tripeptide 33. To a solution of **27** (95 mg, 0.036 mmol) in AcOH (3 mL) was added palladium hydroxide (100 mg) in AcOH and stirred under H_2 (20 atm) for 1 day. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **33** (39 mg, 50%) as a white powder. ESI-TOF-MS (negative) m/z = 834.5 $[M - 2H]^{2-}$; HRMS-ESI FT-ICR (negative): (M) calcd for $C_{69}H_{118}N_{14}O_{33}$, 1670.798; found, 1670.817; ^{1}H NMR (500 MHz, D_2O): δ = 4.86–4.80 (m, 1H, H-1) 4.46–4.40 (m, 3H), 4.36–4.05 (m, 8H, Lac-α-C*H*, Ala-α-C*H*, iGln-α-C*H*, Lys-α-C*H*), 3.86–3.30 (m, 26H), 3.00–2.90 (t, J = 11.4, 4H, Lys-ε-CH_2), 2.37–2.31 (t, J = 9.5, 4H, iGln-γ-CH_2), 2.09–2.0 (m, 4H, iGln-β-CH_2), 2.02–1.81 (m, 18H, NHC(O)CH_3 × 4, Lys-β-C*H* × 2, Lys-δ -C*H* × 2), 1.78–1.69 (m, 2H, Lys-δ -C*H* × 2), 1.58–1.70 (m, 4H, Lys-γ-CH_2 × 2), 1.61–1.4 (m, 2H, Propyl CH_3–CH_2), 1.40–1.35 (m, 6H, Ala-β-CH_3), 1.31–1.28 (m, 6H, Lac-β-CH_3), 0.85–0.80 (t, J = 9.3, 3H, Propyl CH_3).

Tetrasaccharide tetrapeptide **35** and tetrasaccharide pentapeptide **37** was synthesized from **27** in a manner similar to the synthesis of **33**

35. ESI-TOF-MS (negative) m/z = 905.1 $[M - 2H]^{2-}$; HRMS-ESI FT-ICR (negative): (M) calcd for $C_{75}H_{128}N_{16}O_{35}$, 1812.873; found, 1812.896; ^{1}H NMR (500 MHz, D_2O): δ = 4.86–4.80 (m, 1H, H-1), 4.46–4.40 (m, 3H), 4.36–3.95 (m, 10H, Lac-α-C*H*, Ala-α-C*H*, D-iGln-α-C*H*, Lys-α-C*H*), 3.86–3.30 (m, 26H), 3.00–2.90 (t, J = 7.5, 4H, Lys-ε-CH_2), 2.40–2.31 (t, 4H, iGln-γ-CH_2), 2.09–1.82 (m, 22H, iGln-β-CH_2 × 2, NHC(O)CH_3 × 4, Lys-β-CH_2 × 2, Lys-δ -C*H* × 2), 1.78–1.4 (m, 8H, Lys-δ -C*H* × 2, Lys-γ-CH_2 × 2, Propyl CH_3–CH_2), 1.40–1.22 (m, 12H, Ala-β-CH_3 × 4, Lac-β-CH_3 × 2), 0.85–0.80 (m, 3H, Propyl CH_3).

37. ESI-TOF-MS (negative) m/z = 976.64 $[M - 2H]^{2-}$; HRMS-ESI FT-ICR (negative): (M) calcd for $C_{81}H_{138}N_{18}O_{37}$, 1954.947; found, 1954.939; ^{1}H NMR (500 MHz, D_2O): δ = 4.86–4.80 (m, 1H, H-1), 4.46–4.40 (m, 3H), 4.36–4.0 (m, 12H, Lac-α-C*H*, Ala-α-C*H*, iGln-α-C*H*, Lys-α-C*H*), 3.86–3.30 (m, 26H), 2.95–2.91 (t, J = 7.5, 4H, Lys-ε-CH_2), 2.40–2.31 (t, J = 7.0, 4H, iGln-γ-CH_2), 2.09–1.82 (m, 22H, iGln-β-CH_2 × 2, NHC(O)CH_3 × 4, Lys-β-CH_2 × 2, Lys-δ -C*H* × 2), 1.78–1.4 (m, 8H, Lys-δ -C*H* × 2, Lys-γ-CH_2 × 2, Propyl CH_3–CH_2), 1.40–1.22 (m, 12H, Ala-β-CH_3 × 6, Lac-β-CH_3 × 2), 0.85–0.80 (m, 3H, Propyl CH_3).

***N*-Acetylated tetrasaccharide tripeptide 34.** To a solution of **33** (5 mg, 0.025 mmol) in methanol (0.5 mL) were added TEA (10 μL) and Ac_2O (5 μL). After being stirred for 2 h, the mixture was concentrated and the residue was lyophilized from acetonitrile/H_2O to give **34** (5.13 mg, 98%) as white powder. ESI-TOF-MS (negative) m/z = 876.4 $[M - 2H]^{2-}$; HRMS-ESI FT-ICR (negative): (M) calcd for $C_{73}H_{122}N_{14}O_{35}$, 1754.820; found, 1754.848; ^{1}H NMR (500 MHz, D_2O): δ = 4.86–4.80 (m, 1H, H-1), 4.46–4.40 (m, 3H), 4.36–4.05 (m, 8H, Lac-α-C*H*, Ala-α-C*H*, iGln-α-C*H*, Lys-α-C*H*), 3.86–3.30 (m, 26H), 3.10–3.02 (m, 4H, Lys-ε-CH_2), 2.37–2.31 (t, J = 9.5, 4H, iGln-γ-CH_2), 2.09–2.0 (m, 4H, iGln-β-CH_2), 2.02–1.81 (m, 22H, NHC(O)CH_3 × 4, Lys-ε-NHC(O)CH_3 × 2, Lys-β-C*H* × 2, Lys-δ-C*H* × 2), 1.78–1.4 (m, 8H, Lys-δ -C*H* × 2, Lys-γ-CH_2 × 2, Propyl CH_3–CH_2), 1.40–1.35 (m, 6H, Ala-β-CH_3), 1.31–1.24 (m, 6H, Lac-β-CH_3), 0.85–0.80 (t, J = 9.3, 3H, Propyl CH_3).

***N*-Acetylated tetrasaccharide tetrapeptide 36 and *N*-acetylated tetrasaccharide pentapeptide 38 were synthesized from compound 35 (5 mg, 0.025 mmol) in a manner similar to the preparation of 33.**

36. ESI-TOF-MS (negative) m/z = 947.5 $[M–2H]^{2-}$; HRMS-ESI FT-ICR (negative): (M) calcd for $C_{79}H_{132}N_{16}O_{37}$, 1812.873; found, 1812.896; ^{1}H NMR (500 MHz, D_2O): δ = 4.86–4.80 (m, 1H, H-1) 4.46–4.40 (m, 3H), 4.40–4.08 (m, 10H, Lac-α-C*H*, Ala-α-C*H*, D-iGln-α-C*H*, Lys-α-C*H*), 3.90–3.30 (m, 26H), 3.10–3.02 (m, 4H, Lys-ε-CH_2), 2.39–2.18 (m, 4H, iGln-γ-CH_2), 2.09–1.40 (m, 34H, iGln-β-CH_2, NHC(O)CH_3 × 4, Lys-ε-NHC(O)CH_3 × 2, Lys-β-CH_2 × 2, Lys-δ -CH_2 × 2, Lys-δ -C*H* × 2, Lys-γ-CH_2 × 2, Propyl CH_3–CH_2), 1.40–1.22 (m, 12H, Ala-β-CH_3 × 4, Lac-β-CH_3 × 2), 0.85–0.80 (t, J = 9.3, 3H, Propyl CH_3).

38. ESI-TOF-MS (negative) m/z = 1018.5 $[M – 2H]^{2-}$; HRMS-ESI FT-ICR (negative): (M) calcd for $C_{85}H_{142}N_{18}O_{39}$, 2038.968; found, 2038.962; ^{1}H NMR (500 MHz, D_2O): δ = 4.86–4.80 (m, 1H, H-1), 4.46–4.40 (m, 3H), 4.40–4.0 (m, 14H, Lac-α-C*H*, Ala-α-C*H*, D-iGln-α-C*H*, Lys-α-C*H*), 3.90–3.30 (m, 26H), 3.10–3.02 (m, 4H, Lys-ε-CH_2), 2.39–2.30 (m, 4H, iGln-γ-CH_2), 2.09–1.40 (m, 34H, iGln-β-CH_2, NHC(O)CH_3 × 4, Lys-ε-NHC(O)CH_3 × 2, Lys-β-CH_2 × 2, Lys-δ -CH_2 × 2, Lys-δ -C*H* × 2, Lys-γ-CH_2 × 2, Propyl CH_3–CH_2), 1.40–1.20 (m, 18H, Ala-β-CH_3 × 6, Lac-β-CH_3 × 2), 0.85–0.79 (t, J = 7.5, 3H, Propyl CH_3).

Octasaccharide dipeptide 40. To a solution of **29** (140 mg, 0.033 mmol) in acetic acid (3 mL) was added palladium hydroxide on carbon (140 mg) in acetic acid(1 mL) and the mixture was stirred under H_2 (20 atm) for one day. The Pd catalyst was removed by filtration and the filtrate was concentrated. The residue was lyophilized from H_2O to give **40** as a white solid (74 mg, 70%). MALDI-TOF-MS (negative) m/z = 2767.63 $[M - H]^-$; HRMS-ESI FT-ICR (negative): (M) calcd for $C_{111}H_{180}N_{20}O_{61}$, 2769.159; found, 2769.157; ^{1}H NMR (600 MHz, D_2O) δ = 4.94–4.88 (m, 1H), 4.54–4.46 (m, 4H), 4.43–4.24 (m, 16H), 3.94–3.34 (m, 53H),

2.40–2.30 (m, 8H, γ-Gln-CH_2 × 4), 2.19–2.09 (m, 4H, iGln-β−CHH × 4), 2.06–1.90 (m, 28H, NHC(O)CH_3 × 8, iGln-β−CHH × 4), 1.44–1.34 (m, 24H, Ala-β-CH_3 × 4, Lac-β-CH_3 × 4).

Octasaccharide 41. Compound **41** was synthesized from compound **24** in a manner similar to the preparation of **40**.

HRMS-ESI FT-ICR (negative): (M) calcd for $C_{79}H_{128}N_8O_{49}$, 1972.777; found, 1972.773; ^{1}H NMR (600 MHz, D_2O) δ = 5.23–5.21 (m, 1H), 4.54–4.46 (m, 4H), 4.43–3.92 (m, 56H), 1.99–2.10 (m, 24H, NHC(O)CH_3 × 8), 1.21–1.40 (m, 12H, Lac-β-CH_3, CH_3–CH_2–OCO × 4); ^{13}C NMR (150 MHz, D_2O) δ = 182.1, 181.4, 174.8, 174.5, 174.4, 174.2, 105.5, 102.2, 100.1, 79.9, 79.1, 78.8, 77.4, 76.1, 75.8, 75.3, 78.2, 74.9, 73.4, 72.0, 71.3, 70.3, 69.7, 61.2, 60.5, 59.6, 56.1, 55.4, 54.6, 54.3, 22.3, 22.0, 21.6, 18.4.

Stimulation of human mononuclear cells (MNC)

MNC were isolated from heparinized blood of healthy adult donors by Ficoll-Isopaque density gradient centrifugation.[29] Isolated MNC were washed in PBS and cultured in RPMI 1640 containing 10% human pooled serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Biochrom, Berlin, Germany). Cells (1 × 10^6/ml) were stimulated in duplicates with various amounts of sPG (kind gift from Prof. U. Zähringer, Research Center Borstel, Borstel, Germany), synthetic peptidoglycan fragments or LPS from *S. enterica serovar friedenau* (kind gift from Prof. H. Brade, Research Center Borstel, Borstel, Germany). In blocking experiments, cells were preincubated for 30 min with 10 μg ml^{-1} of the anti-CD14 mAb MEM-18,[30] the LPS antagonist compound 406, or the anti-TLR2 mAb clone 2392 (Genentech, CA, USA). After 4 h, supernatants were collected and the content of TNF-α was quantified by ELISA (Biosource).

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